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Expression of a functional α-macroglobulin receptor binding domain in *Escherichia coli*

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We have expressed receptor-binding domains of human α_2-macroglobulin and rat α_1-macroglobulin in *Escherichia coli* Expression levels of both recombinants were quite high, but the human one was insoluble, probably forming inclusion bodies. The rat domain, which lacks the human disulfide, was produced in a soluble form and readily purified by two simple chromatographic steps Purified recombinant rat α_1-macroglobulin receptor-binding domain was fully functional in binding to the α-macroglobulin receptor on human fibroblasts. This 142 residue domain should serve as an excellent template for analyzing the structural requirements for α-macroglobulin receptor ligation and dissecting the varied biological functions resulting from such ligation

α-Macroglobulin; Protein expression; Lipoprotein receptor

1. INTRODUCTION

α-Macroglobulins (αMs), such as human α_2-macroglobulin (α_2M) and rat α_1-macroglobulin (α_1M), probably constitute a general proteinase clearance system and backup to the more specific proteinase inhibitors present in mammals [1]. Related to the αMs, although with distinct functions, are the complement components, C3 and C4. Although they probably diverged during early chordate evolution [2], certain properties reveal that C3, C4 and the αMs descended from a common ancestor. These properties include primary sequence homology, activation by endopeptidases in a region equidistant from the terminals of the precursor protein chains, and the presence of an intra-chain β-cysteinyl-γ-glutamyl thiol ester about one third of the distance from the C-terminus (reviewed by Sottrup-Jensen [3]). In modern vertebrates, C3 and C4 participate in proinflammatory and cytolytic reactions in response to infectious agents [4].

Following reaction with proteinases, the complement components and most αMs undergo a conformational change that results in exposure of a receptor recognition site. Receptor ligation allows C3- and C4-coated particles to bind blood cells to aid in immune complex clearance and promote phagocytosis. The receptor that recognizes the proteinase-inhibitory αMs has a much broader cell-type distribution (for review see Pizzo [5]). Although initially thought only to be responsible for removal of αM–proteinase complexes, ligation of the receptor may also influence cellular functions [6–8].

The human αM receptor has been cloned and sequenced [9,10], and is identical to one previously reported as the low density lipoprotein receptor-related protein (LRP). The LRP receptor is responsible for binding apolipoprotein E-enriched β-migrating very low density lipoproteins [11], although this receptor may not be the primary one for uptake of β-migrating very low density lipoproteins in vivo [12]. Very recently, the αM/LRP receptor has also been shown to bind *Pseudomonas* exotoxin A [13] and tissue plasminogen activator, either alone or via its complex with a specific tissue inhibitor [14,15]. It thus seems that this single receptor is capable of binding and internalizing several distinct ligands, none of which appear to show significant sequence homology, and most of which probably bind to different sites on the receptor.

αMs derived from various mammals show comparable binding affinities for mouse and human receptors, suggesting that the specificity of the interaction is highly conserved. A 20–30 kDa C-terminal fragment derived from the 180 kDa parent chain of αMs contains most, if not all, of the information required for binding to the cellular receptor [16–19]. Although C3 and C4 are homologous to αMs, they utilize different regions of their polypeptide chains for receptor binding. Moreover, complement receptors 2 and 3 recognize primary sequence epitopes whereas binding of αMs to their receptor is dependent on the correct folding of the ligand [3,4]. Consequently, peptide-based strategies that have been employed to characterize the complement receptor-binding epitopes are unlikely to succeed in defining

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the αM-receptor binding site. To characterize the region of αMs responsible for recognizing the receptor we are expressing small regions of the parent molecules in forms that preserve binding activity. These can serve as templates for mutagenesis strategies and allow dissection of the biological effects of αM receptor ligation.

In the present study, we report the cloning and expression of the C-terminal receptor binding domain from rat α_1M. Our use of rat α_1M, rather than another αM, is based on the following two principles. Firstly, this is the only known αM devoid of the 1,329–1,444 disulfide [20] (human α_2M numbering system). Secondly, a fragment of 40 kDa isolated from rat α_1M, which contains the C-terminal receptor binding site, had previously been shown to regain activity following dialysis from 6 M guanidinium chloride [19]. Consequently, folding should be spontaneous and not be dependent on disulfide formation.

2. EXPERIMENTAL

2.1 *Materials*

Escherichia coli TG1 [21] was used for cloning and expression. Restriction endonucleases, T4 ligase, T4 polynucleotide kinase, helper phage MK107, and pFlag-1 vector were purchased from International Biotechnologies Inc., New Haven, CT. Sequenase version 2.0 was purchased from United States Biochemicals, Cleveland, OH, and α-[^{35}S]thio dATP (1,200 Ci/mmol) was from Dupont, Wilmington, DE. HS27 human skin fibroblasts (ATCC catalog number CRL 1634) were the gift of Michael Banda, University of California at San Francisco

2.2. *Construction of expression vector*

All DNA manipulations were performed as described [21]. The following two primers with embedded *Hin*dIII recognition sites (underlined): 5′-CAAAGCTTGAGGCAGAAGGAGAAGCG-3′, and 5′-CGAAGCTTTTCACGTGTGGAGGAGTC-3′ were used to amplify a 484 bp fragment coding for the C-terminal region of rat α_1M cDNA [20]. Amplification was performed by polymerase chain reaction (PCR) employing *Taq* polymerase and reagent kits from Perkin-Elmer/Cetus, Emeryville, CA, in a Perkin-Elmer DNA thermal cycler. The amplified product was purified from a 1% low gelling temperature agarose gel (FMC BioProducts, Rockland, ME), cut with *Hin*dIII then ligated into the *Hin*dIII site of pFlag-1. The ligated product was used to transform *E. coli* TG1 by the procedure of Hanahan [22]. The plasmid construct was characterized by endonuclease digestion and nucleotide sequence analysis [23]. To obtain single-stranded DNA for sequencing, the transformant was co-infected with helper phage MK107 according to the supplier's instructions.

2.3. *Site-directed mutagenesis*

Bases 105–188 of the vector (encoding the OmpA signal peptide and the trypsinogen activation peptide) were deleted with the mutagenic primer: 5′-GGGTGCTTCTCCTTCCATATGATATCTCCTG-3′. Mutagenesis was carried out as described [21] using single-strand template obtained with helper phage and Sequenase version 2.0 as the extending polymerase. Transformants were screened by PCR to detect the deletion, and one candidate, termed $p\alpha_1$M-RBD, was sequenced in its entirety to confirm the precision of the deletion.

2.4. *Expression and purification of rat α_1M receptor binding domain (RBD)*

E. coli TG1 transformed with $p\alpha_1$M-RBD was grown in 2 l of 2× TY containing 50 μg/ml ampicillin. When the $A_{600\ nm}$ of the culture reached 0.5 the cells were induced with isopropyl thiogalactopyranoside (IPTG) to a final concentration of 1 mM for 3 h then harvested by centrifugation at 13,000×g for 30 min. The cell pellet was resuspended in 300 ml ice-cold 50 mM Tris-Cl buffer, pH 8.0, then lysed by sonication (Sonics and Materials Inc., Danbury, CT) for two 5 min bursts. The lysate was centrifuged at 13,000×g for 40 min and the supernatant was loaded onto a 60 ml Q Sepharose FF ion exchange column (Pharmacia) equilibrated with 50 mM Tris-Cl, pH 8.0. The rat α_1M-RBD was eluted with a linear gradient from 0 to 1 M NaCl in 50 mM Tris-Cl, pH 8.0. Fractions were monitored by SDS-PAGE under reducing conditions.

Fractions containing the bulk of the rat α_1M-RBD were pooled and concentrated from 30 ml to 1.5 ml by ultrafiltration (Amicon, Danvers, MA) with a 10 kDa cut-off membrane (YM10). In addition, the buffer was exchanged with 1× Hank's salt solution buffered with 10 mM HEPES, pH 7.4 (binding buffer). The protein was further purified on a 25 ml Superose 12 gel filtration column (Pharmacia) equilibrated with the same buffer. The peak containing α_1M-RBD from the gel filtration column was used directly for cell binding studies.

2.5 *Amino acid sequence and compositional analysis*

Automated Edman degradation was carried out in an Applied Biosystems 477A sequencer with on-line analysis of phenylthiohydantoins using an Applied Biosystems 120A HPLC. Amino acid compositional analysis was carried out in a Beckman 6300 amino acid analyzer. A sample of desalted α_1M-RBD was hydrolyzed in 6 N HCl for 24 h at 110°C in vacuo. All residue weights were calculated by integration, with the exception of Pro and Trp. The weight of the protein sample, allowing calculation of the extinction coefficient ($A_{280}^{1\%, 1\ cm}$), was calculated as the sum of the weights of each residue. The extinction coefficient of the purified recombinant protein was 11.5.

2.6. *Mass spectrometry*

A sample of purified recombinant rat α_1M-RBD was analyzed by Stanley Hefta, Beckman Research Institute, Duarte, CA, using electrospray-ionization mass spectrometry [24]. Briefly, the sample was introduced into a Finnegan TSQ-700 quadropole mass spectrometer by reverse-phase liquid chromatography. The averaged multiple charged spectrum was collected over the scan range of 50–2000 AMU and the mass of the protein was assigned by using Finnegan-MAT BIOMASS software.

2.7. *Cellular receptor binding assay*

The conformational change induced by reaction of αMs with proteinases also occurs when many αMs react with small primary amines such as methylamine. Thus, methylamine-treated αMs are often used to imitate proteinase inhibitor complexes for studying receptor binding and ancillary events, obviating problems caused by introducing proteinases into cell binding experiments. Therefore, receptor-recognized human α_2M was made by incubation overnight in 0.2 M methylamine hydrochloride, pH 8.0, at a protein concentration of 4 mg/ml. Cell binding experiments were performed essentially as previously described [18], except that human fibroblasts were substituted for mouse macrophages. HS27 cells were seeded on 24-well culture plates at a density of 20,000 cells per well in DMEM containing 10% fetal bovine serum and grown for 48 h until about 80% confluent. The cells were washed twice with binding buffer at 4°C and incubated with various concentrations of unlabeled competing ligand (methylamine-treated human α_2M, or rat α_1M-RBD) containing 0.3 nM ^{125}I-labeled human α_2M-methylamine in binding buffer with 1% BSA. After 6 h at 4°C the cells were washed once with binding buffer containing 1% BSA, and twice with binding buffer alone. Cells were removed from the wells following lysis in 0.5 ml 0.1 M NaOH and radioactivity counted in a γ-counter to determine the extent of bound radioligand. Non-specific binding was determined as the amount of radioligand bound in the presence of 5 mM EDTA. All data points were measured at least three times and the results averaged. The I_{50} is the concentration of ligand required to decrease the specific binding of radioligand by 50%. Under the conditions employed in this study, where t

radioligand tracer is used at its K_d, the K_d of competing ligands is calculated according to the relationship, $I_{50}=2K_d$, the use of which with this system was detailed in an earlier publication [18].

3. RESULTS AND DISCUSSION

3.1. *Construction of a rat α_1M-RBD expression vector*

Our initial strategy was to drive expression of an OmpA signal peptide/trypsinogen activation peptide/α_1M-RBD fusion so that the protein would be secreted following OmpA removal to provide for facile purification using antisera raised against the activation peptide. Recombinants were isolated and the correct orientation of the α_1M-RBD insert was determined by restriction endonuclease digestion of plasmid minipreps and nucleotide sequencing. One recombinant was chosen for expression by induction with IPTG as described in section 2. This clone produced a product that was insoluble unless dissolved in 6 M guanidinium chloride, and which precipitated upon dialysis into 5 mM Tris-Cl buffer, pH 8, containing 0.05 mM EDTA. The product was sequenced and found to contain the OmpA signal peptide and the trypsinogen activation peptide, indicating lack of the expected signal peptide processing. We suspect that the hydrophobicity of the OmpA signal peptide prevented proper folding, and promoted aggregation and precipitation of the recombinant rat α_1M-RBD. Since expression was at such high levels, we decided to utilize the same expression vector, but to delete the OmpA signal peptide and the trypsinogen activation peptide from the recombinant vector and to express the protein devoid of the N-terminal extensions provided by the parent vector.

Deletion was accomplished with a 31-mer mutagenic oligonucleotide primer complementary to sequences flanking the OmpA signal peptide and the trypsinogen activation peptide, thus looping out 83 bp. PCR analysis of 24 colonies revealed 8 with deletions. One mutant was sequenced and was found to have the engineered deletion and no other mutations. This construct was designated *p*α_1M-RBD (Fig. 1). The initiator methionine provided by the vector is followed immediately by Glu^{1334} (numbering of Eggerston et al. [20]), which is the N-terminal residue of the C-terminal α_1M receptor binding domain [19].

3.2. *Expression and purification*

The α_1M-RBD expressed in *E. coli* was only visualized following induction with IPTG and was the second most abundant protein seen by Coomassie staining (Fig. 2). The protein migrated with an apparent size of 22 kDa in reduced SDS-PAGE, rather than the 30 kDa size of the same fragment from a digest of natural rat α_1M (see [19]). When we attempted expression of a human α_2M-RBD utilizing the same procedure, the product was insoluble whether or not it contained the OmpA/trypsinogen activation peptide. Preliminary experiments confirmed that the rat α_1M-RBD was soluble, so we concentrated our efforts on purifying and characterizing this recombinant domain. Although the receptor binding fragment from the digest of natural rat α_1M has previously been designated 20 kDa, its size is closer to 30 kDa in reduced SDS-PAGE, so we will refer to it as the 30 kDa fragment. The smaller size of the

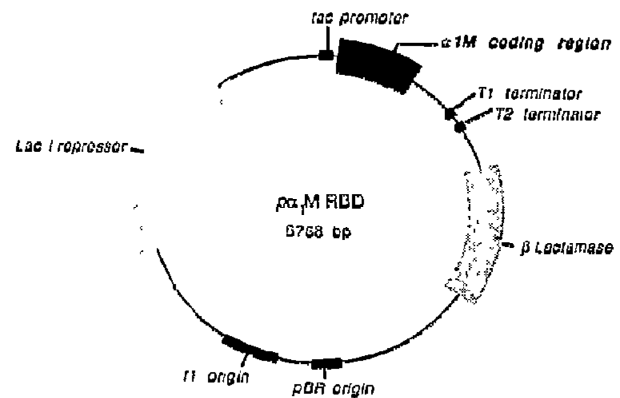

Fig. 1. Expression vector *p*α_1M-RBD. The construct is derived from pFlag-1 with the α_1M-RBD coding region ligated into the *Hin*dIII site of the multiple cloning region. The OmpA signal peptide and trypsinogen activation peptide of the parent vector were deleted by site-directed mutagenesis. Regions of the vector important in cloning and expression are indicated; further details of the vector may be obtained from the distributors (see section 2).

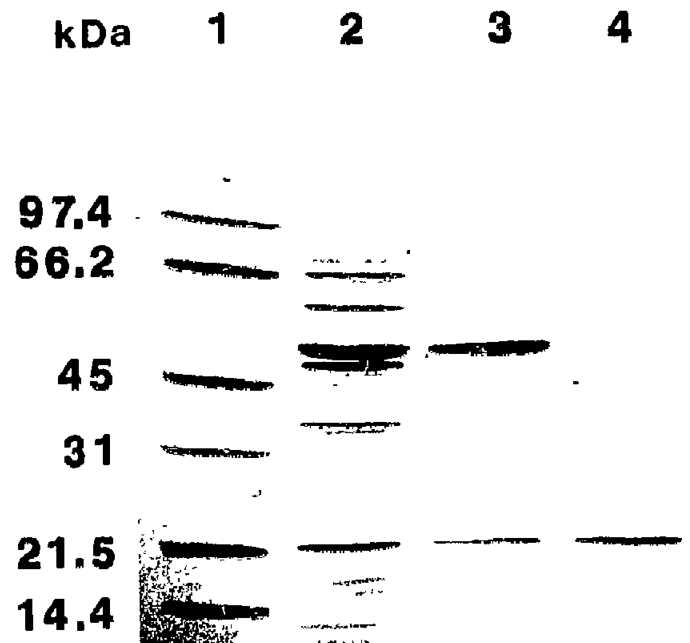

Fig. 2. Purification of recombinant rat α_1M-RBD. All samples were boiled for 5 min in 1% SDS and 20 mM DTT and run in a 5–15% linear gradient SDS-PAGE gel as described by [27]. Lane 1, standard proteins, lane 2, 40 μl from 300 ml of crude lysate; lane 3, 10 μl from a 30 ml pool of Q-Sepharose FF eluate; lane 4, 2 μl from 3 ml of Superose 12 peak. Since lanes 2 and 4 show about the same amount of rat α_1M-RBD, we estimated the yield to be around 20%.

recombinant α_1M-RBD is probably due to the lack of carbohydrate.

During ion exchange on Q-Sepharose, the α_1M-RBD eluted in a sharp peak that was contaminated with a 50 kDa protein in addition to various other high molecular weight proteins (Fig. 2, lane 3). These contaminants were removed by gel filtration on a Superose 12 column; the protein was over 90% pure as visually quantitated by Coomassie blue staining, with a yield of about 20%. The α_1M-RBD behaved as a single homogeneous species during both chromatography steps. The first 15 residues of the purified α_1M-RBD were confirmed by Edman degradation, and the mass of the protein was 16,179 Da by mass spectrometry. This is in excellent agreement with the expected mass of 16,180 Da calculated from the residue composition of the domain, and confirms the authenticity of the product.

3.3. *Receptor binding activity*

The α_1M-RBD competed with [^{125}I]methylamine-treated human α_2M for binding to fibroblasts, as has previously been shown for the natural 30 kDa fragment, implying recognition by the α_2M receptor. The K_d of the recombinant α_1M-RBD was 20 nM, lower than the value of 62 nM for the binding of natural 30 kDa fragment to mouse macrophages, (which was previously calculated as 125 nM based on an assumed extinction coefficient of 0.5 [16,19]). This difference may reflect species differences of the receptor.

There is approximately a two-order of magnitude difference in binding of α_1M-RBD and natural α_2M to the receptor (Fig. 3). This is consistent with previous results [19,25], and has two likely explanations; (i) αMs (which are usually tetramers) bind by occupying two receptors, thus lowering the observed K_d [25] with respect to the monomeric RBD; (ii) not all of the receptor binding site is contained in the RBD fragment [19]. We do not know which of these speculations is correct, but the ability to express functional αM domains gives us the opportunity to test them.

We conclude that the recombinant rat α_1M-RBD and the 30 kDa fragment of natural rat α_1M are close enough in their affinities that they can be thought of as equivalent in their ability to bind to the human receptor. Their similar affinities confirms earlier suggestions [26] that carbohydrate is not important in binding of α-macroglobulins to their receptor.

Although the rat α_1M-RBD shares 60–75% identity with the equivalent regions of other αMs, it lacks the two cysteines present in the latter domains. Correct folding of the cysteine-containing RBDs probably requires disulfide formation, since we found that the recombinant human α_2M-RBD was insoluble, and since Sottrup-Jensen et al. [16] found that fragmentation, denaturation or reduction of the natural human α_2M receptor-binding fragment abolished its activity. Presumably, the rat α_1M-RBD is able to fold spontaneously

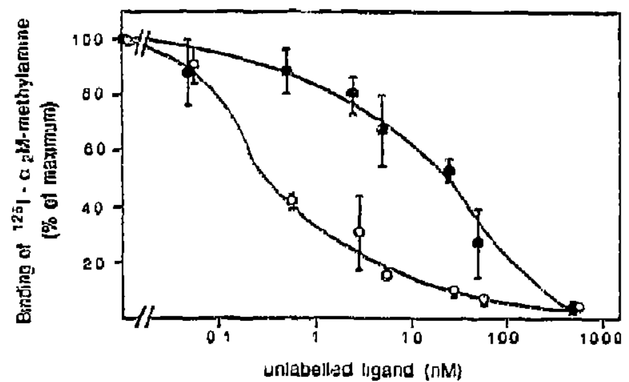

Fig. 3. Binding of recombinant rat α_1M-RBD to human fibroblasts. The ability of recombinant rat α_1M-RBD (●) to prevent binding of ^{125}I-labeled human α_2M-methylamine is compared to that of unlabeled human α_2M-methylamine (○). Error bars are standard deviations from the mean using at least three data points per ligand concentration. One hundred percent binding was 771 cpm above a background of 100 cpm

without the formation of a disulfide, thus facilitating production of a soluble functional domain in *E. coli.* The ready solubility and high level of expression of the rat α_1M-RBD makes it an excellent candidate for studies, such as site-directed mutagenesis, designed to detail the mechanism and specificity by which the receptor recognizes α-macroglobulins.

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